APOPTOSIS INHIBITION BY ADENOVIRUS E3/6.7K

FIELD OF THE INVENTION

This application relates to inhibition of apoptosis.

BACKGROUND OF THE INVENTION

Viral infection is a cellular injury, and it results in the induction of programmed cell death of the host cell. Many viruses, particularly persistent DNA viruses modify the apoptotic response of a cell to allow continued virus replication. Apoptosis can be induced by the members of the TNF receptor super-family such as Fas (APO-1 or CD95) and p55 Tumor Necrosis Factor Receptor (p55 TNFR) as well as the death domain-containing receptors 3, 4 and 5 (DR3, DR4 and DR5, respectively). The intracellular factors responsible for death of the cell are highly conserved across species and are the target of viral inhibitors of apoptosis.

It appears that proteins belonging to very different classes of virus have evolved to block the same cellular apoptotic event. This convergent evolution is evidenced by the classification of viral inhibitors of apoptosis. For example, adenovirus E1B 55K (Debbas and White, 1993), SV40 Large T antigen(Levine, 1997) (Lill *et al.*, 1997) and human papilloma virus E6 (Levine, 1997) inhibit p53-mediated lysis. The cellular survival factor Bcl-2 is mimicked by adenovirus E1B 19K (White, 1996), Epstein-Barr virus BHRF1 (Henderson *et al.*, 1993) and African swine fever virus LMW5-HL (Neilan *et al.*, 1993). Members of the Interleukin 1b Converting Enzymes (ICE)-family of terminal proteolytic enzymes, also known as caspases, are blocked by baculovirus p35 (Clem *et al.*, 1991)(Xue and Horvitz, 1995) and crmA, the cowpox serpin protein (Zhou *et al.*, 1997) (Tewari and Dixit, 1995). The adenovirus E3/10.4K and E3/14.5K downregulate surface Fas (Elsing and Burgert, 1998) (Tollefson *et al.*, 1998) (Shisler *et al.*, 1996), while the Inhibitors of Apoptosis (IAP) family of baculovirus and mammalian homologues interact with the TNF-α receptor associated factors (TRAFs) therefore blocking the signalling cascade that leads to the recruitment of caspases (Liston *et al.*, 1996) (Duckett *et al.*, 1996) (Deveraux *et al.*, 1997). The activation of FADD-like interleukin-1beta-converting enzyme (FLICE), also known as caspase-8, through Fas is blocked by viral-FLICE-inhibitory proteins (vFLIPs), found in the genomes of various

types of herpesvirus (Thome *et al.*, 1997) and by the E3/14.7K of adenovirus (Chen *et al.*, 1998).

Adenovirus (Ad) is a very common human pathogen that results in persistent infections of the respiratory or gastrointestinal tract (Fox *et al.*, 1969) (Fox *et al.*, 1977). Persistent infections stem from an elaborate evasion of the host defense mechanisms. The adenovirus genes responsible for immune evasion map to the Early 3 (E3) region of the Ad genome (Wold and Gooding, 1989). The persistence, ease of infection and weak pathogenesis have made adenovirus suitable as vectors for gene therapy. Currently, Ad gene transfer vectors are the most efficient technique available for *in vivo* gene transduction. The size of the transduced DNA that can be accommodated by adenovirus is greater than 30kbp greatly surpassing all other viral systems. In the case of Ad vectors the genetic makeup of the original vectors was designed to accommodate large fragments of DNA for the transduced gene, to the expense of areas of the adenoviral genome that were considered dispensable. The E3 region was one of the first areas to be replaced.

The 6.7K protein encoded by the E3 region (E3/6.7K) sequence does not have any significant homology to any other known proteins. It is well conserved between group C Ad2 and Ad5 adenovirus and between group B Ad3, Ad7 and Ad35, adenovirus (Hawkins *et al.*, 1995). The Ad2 E3/6.7K protein (Wilson-Rawls *et al.*, 1990) has been shown to be an integral membrane protein localized to the endoplasmic reticulum (ER) (Wilson-Rawls and Wold, 1993). The protein is present in two forms, one unglycosylated with an apparent molecular weight of 8kDa and one glycosylated with an apparent weight of 14kDa. The protein, though targeted to the ER, does not have a cleavable signal sequence, but it has a hydrophobic central region that could act as a signal anchor (Wilson-Rawls *et al.*, 1994).

The major impediment for the success of Ad vectors as well as all the other gene transfer technologies is the unexpectedly strong immune response to cells infected by a modified Adenovirus. The strong immune response to modified Ad vectors is mediated by the circulating cytokine Tumor Necrosis Factor (TNF) α (Elkon *et al.*, 1997) and by the innate immune response (Worgall *et al.*, 1997). The negative effects of an immune response might be alleviated by implementing immunomodulatory proteins that allow the vector and the transduced cells to survive the immune response (Zhang *et al.*, 1998).

The evasion of immune response is also a central impediment to the establishment of successful transplant technology as well as the treatment of autoimmune and

neurodegenerative diseases. Apoptosis of the affected organ is often the result of neurodegenerative inflammatory disease. Factors that prevent apoptosis could lead to better therapies for these conditions.

Cell culture reactor expression systems are limited only by the ability of cells to grow and produce proteins of commercial or medical interest (Singh and al-Rubeai, 1998) (al-Rubeai, 1998). As cell grow they reach densities where protein production stops and producer cells undergo apoptosis in response to factors that are currently poorly characterized (al-Rubeai and Singh, 1998). There is potential for improving protein yield by avoiding the apoptotic response of cells grown in culture by including an antiapoptotic protein in the makeup of the cell (Simpson *et al.*, 1998).

SUMMARY OF THE INVENTION

As now shown herein, TNF-α induced apoptosis and TNF-α induced release of arachidonic acid are significantly reduced in cells expressing transfected E3/6.7K. It is now shown that the mechanism of E3/6.7K involves the cleavage and inactivation of cytosolic phospholipase A2 (cPLA2). This enzyme is important in the generation of proinflammatory agents and is involved in release of arachidonic acid. E3/6.7K has no sequence homology to any of the previously described inhibitors of apoptosis. E3/6.7K therefore represents a new class of viral inhibitors of apoptosis localized to the endoplasmic reticulum.

This invention provides methods for immune evasion and for evasion of apoptosis by implementing the E3/6.7K protein from adenovirus.

This invention also provides vectors containing the adenovirus E3/6.7K region for use in gene therapy or to minimize transplant rejection. This invention also provides methods for improving protein yield from cell culture.

This invention provides a method for inhibiting apoptosis of a cell comprising treating the cell, a mammal comprising the cell, or a tissue comprising the cell, with an effective amount a E3/6.7K polypeptide. The treating step may comprise administering a nucleic acid encoding the polypeptide whereby the polypeptide is expressed in the cell. The administering may be by a viral vector comprising the nucleic acid, with the proviso that if the vector is adenovirus, the nucleic acid is other than a naturally occurring nucleic acid from E3 of

adenovirus, or the nucleic acid is under the transcription control of a promotor not found in adenovirus.

The method of this invention may be for treatment of a mammalian patient suffering from a degenerative (e.g. neurodegenerative) disease, an immunodeficiency, or an inflammatory disease as a result of which disease, cellular apoptosis occurs.

This invention also provides a method of decreasing apoptosis in a tissue or cell population in a patient comprising: (a) withdrawing tissue or a cell from the patient, (b) treating the tissue or cells with an effective amount of a E3/6.7K polypeptide; and (c) returning the treated tissue or cells to the patient. The cell population may comprise or consist of leukocytes.

This invention also provides a pharmaceutical composition comprising a E3/6.7K polypeptide and a carrier suitable for facilitating delivery of the polypeptide to a cell, as well as a nucleic acid comprising a non-naturally occurring adenovirus E3 nucleic acid capable of encoding a E3/6.7K polypeptide.

This invention also provides a recombinant virus comprising a nucleic acid encoding a E3/6.7K polypeptide with the proviso that if the virus is adenovirus, the nucleic acid is other than a naturally occurring adenovirus E3 nucleic acid or the nucleic acid is under the transcriptional control of a promoter, not from adenovirus.

This invention also provides the use of a E3/6.7K polypeptide, a nucleic acid encoding said polypeptide or a vector comprising said nucleic acid for the treatment of apoptosis, and the use of a E3/6.7K polypeptide, a nucleic acid encoding said polypeptide or a vector comprising said nucleic acid for the preparation of a medicament for the treatment of apoptosis.

This invention also provides an assay for an agent that modulates anti-apopotoic activity of a E3/6.7K polypeptide which comprises: combining the polypeptide with a sample suspected of comprising the agent; and, determining whether anti-apoptotic activity is modulated. The combining may be in a cell or an extract of a cell that is rescued from apoptosis by an E3/6.7K polypeptide that is expressed in or is administered to the cell. The determining may be by detection of or measurement of TNF-α activity, such as arachidonic acid release.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Nucleic acid sequence showing the alignment of the naturally occurring nucleic acid sequence(SEQ ID NO: 1), which is capable of encoding a E3/6.7K protein corresponding to that of Adenovirus serotype Ad2 wild-type(Wt.), and the Polymerase Chain Reaction (PCR) nucleic acid product expected (SEQ ID NO: 2) when the forward primer(FP - SEQ ID NO: 3) and reverse primer(RP - SEQ ID NO: 4) are used to amplify the wild-type sequence(Wt.). Start codons are underlined. The nucleic acids shown in **bold** in the forward primer(FP) represent a modification to provide a Kozak consensus sequence. The nucleic acids shown in **bold** in the reverse primer(RP) is a modified stop codon to enhance translation.

Figure 2 Amino acid sequence showing the alignment of the E3/6.7K protein amino sequences from the Ad2(SEQ ID NO: 4) and Ad5(SEQ ID NO: 5) Adenovirus serotypes. The Ad 2 E3/6.7K amino acid sequence is 61 amino acids in length and the Ad5 E3/6.7K amino acid sequence is 63 amino acids in length.

DETAILED DESCRIPTION OF THE INVENTION

As used herein for description of this invention, the terms E3/6.7K protein and E3/6.7K polypeptide include: a protein or fragment thereof encoded by a nucleic acid as depicted in Figure 1; a Ad2 or Ad5 adenovirus serotype protein or fragment thereof as depicted in Figure 2; or a protein having at least 70% similarity as defined by a Basic Blast search using default parameters to the Ad2 or Ad5 proteins depicted in Figure 2. The Ad5 protein is actually about 7.1K.

Modulation of apoptosis, including inhibition of apoptosis or rescue of a cell from apoptosis may be determined by various methods known in the art, including assays which directly measure apoptosis or which measure the activity of TNF-α, such as those described herein.

Gene Therapy Methods. The isolated nucleic acid molecule depicted in Figure 1, a nucleic acid molecule encoding an E3/6.7K protein as defined herein or a nucleic acid molecule complementary to those described above, may be incorporated into a vector suitable

for introducing the nucleic acid into cells of a mammal to be treated, to form a transfection vector. Suitable vectors for this purpose include retroviruses and adenoviruses.

Techniques for the formation of the transfection vector comprising a E3/6.7K-encoding nucleic acid molecule are well-known in the art, and are generally described in "Working Toward Human Gene Therapy," Chapter 28 in Recombinant DNA, 2nd Ed , Watson, J.D. ci al., eds., New York: Scientific American Books, pp. 567-581 (1992), and in the references cited therein.

Analogous gene therapy approaches have proven effective or to have promise in the treatment of other mammalian diseases such as cystic fibrosis (Drumm, M.L. *et al.*, Cell 62: 1227-1233 (1990); Gregory, R.J. *et al.*, Nature 347:358-363 (1990); Rich, D.P. *et al.*, Nature 347:358-363 (1990)), Gaucher disease (Sorge, J. el al., Proc. Natl. Acad. Sci. USA 84:906-909 (1987); Fink, J.K. ci al., Proc. Natl. Acad Sci. USA 87:2334-2338 (1990)), certain forms of hemophilia (Bontempo, F.A. petal, Blood 69:1721-1724(1987); Palmer, T.D. *et al.*, Blood 73:438-445 (1989); Axelrod, J.H. *et al.*, Proc. Nail. Acad. Sci. USA 87:5173-5177 (1990); Armentano, D. *et al.*, Proc. Natl. Acad. Sci. USA 87:6141-6145(1990)) and muscular dystrophy (Partridge, T.A. ci aL, Nature 337:176-179(1989); Law, P.K. *et al.*, Lancet 336:114-115 (1990); Morgan, J.E. el al., J. Cell Biol. 111:2437-2449 (1990)), as well as in the treatment of certain cancers such as metastatic melanoma (Rosenberg, S.A. *et al.*, Science 233:1318-1321(1986); Rosenberg, S.A. *et al.*, N. Eng. J. Med. 319:1676-1680 (1988); Rosenberg, S.A. *et al.*, N. Eng. J. Mcd. 323:570-578 (1990)). Various promoters may be used to enhance gene expression in specific tissues. For example, in neuronal tissue the neuron-specific enolase promoter (Ad-NSE) and in Lyphocytes the lck promoter could be used.

Organ Transplant Methods. E3/6.7K has potential uses in tissue and organ transplantation to render them less susceptible to apoptosis. In particular, it can be used to genetically modify endothelial or other mammalian cells to render them capable of expressing E3/6.7K protein, which specifically inhibits TNF-α induced apoptosis in transfected cells. It may also be used in the transplantation of genetically modified cells, or tissue or organs comprising such cells, capable of expressing the inhibiting protein (E3/6.7K); it most particularly is directed to methods of transplanting modified xenogeneic or allogeneic cells, tissue or organs; recombinant genes, proteins and vectors for accomplishing same; and the

cells, tissue or organs, as well as non-human transgenic or somatic recombinant animals, so modified.

Appropriate methods of inserting foreign cells or DNA into animal tissue include microinjection, embryonic stem (ES) cell manipulation, electroporation, cell gun, transfection-k, transduction, retroviral infection, etc. Genes can be inserted into germ cells (eg. fertilized ova) to produce transgenic non-human animals bearing the gene, which is then passed on to offspring.

Genes can also be inserted into somatic/body cells to provide somatic recombinants, from whom the gene is not passed on to offspring.

In one embodiment, gene transcription is subject to an inducible promoter, so that expression of the recombinant protein can be delayed for a suitable period of time prior to grafting. In another embodiment, the gene is inserted into a particular locus, eg. the thrombomodulin or P-selectin locus. Subsequently, the construct is introduced into embryonic stem (ES) cells, and the resulting progeny express the construct in their vascular endothelium.

For gene delivery, retroviral vectors, and in particular replication-defective retroviral vectors lacking one or more of the gag, pol, and env sequences required for retroviral replication, are well-known to the art and may be used to transform endothelial cells.

The ability of adenoviruses to attach to cells at low ambient temperatures is an advantage in the transplant setting which, can facilitate gene transfer during cold preservation. Alternative means of targeted gene delivery comprise DNA-protein conjugates, liposomes, etc.

Cells or cell populations can be treated in accordance with the present invention *in vivo* or *in vitro*. For example, for purposes of *in vivo* treatments, p65RHD vectors can be inserted by direct infection of cells, tissues or organs in situ. For example, the vessels of an organ such as a kidney can be temporarily clamped off from the blood circulation, and the blood vessels perfused with a solution comprising a transmissible vector construct containing the E3/6.7K gene for a time sufficient for the gene to be inserted into cells of the organ; and on removal of the clamps, blood flow can then be restored to the organ and its normal functioning resumed.

In another embodiment, cell modification can be carried out *ex vivo*. Cell populations can be removed from the donor or patient, genetically modified by insertion of vector DNA,

and then implanted into the patient or a syngeneic or allogeneic recipient. For example, an organ can be removed from a donor, subjected *ex vivo* to the perfusion step described above, and the organ can be re-grafted into the donor or implanted into a different recipient of the same or different species.

Preferably the protein encoding region is under the control of a constitutive or inducible promoter. An advantage of employing an inducible promoter for transplantation purposes is that the desired high level transcription/expression of the active gene/protein can be delayed for a suitable period of time before grafting. For example, transcription can be obtained on demand in response to a predetermined stimulus, such as, eg. the presence of tetracycline in the cellular environment. An example of a tetracycline-inducible promoter which is suitable for use in the invention is disclosed by Furte *et al.*, PEAS US 91 (1994) 9302-9306. Alternatively, a promoter system where transcription is initiated by the withdrawal of tetracycline is described by Gossen and Bujard, PEAS URSA 90 (1992) 5547-51.

The following terms are referred to in this section:

"Allogeneic" means that the donor and recipient being of the same species.

"syngeneic" means that the condition wherein donor and recipient are genetically identical.

"Autologous" means that donor and recipient are the same individual. "Xenogeneic" and "xenograft" means that the condition where the graft donor and recipient are of different species.

Methods for peptide preparation, expression and administration. A polypeptide according to the invention or a derivative thereof may be administered as a pharmaceutical composition which may be formulated according to various methods. For example, such a formulation may be a solution or suspension. However, as is well known, peptides can also be formulated for therapeutic administration as tablets, pills, capsules, sustained release formulations or powders. The preparation of therapeutic compositions which comprise polypeptides as active ingredients is well understood in the art. Typically, such compositions are prepared in injectable form, eg. as liquid solutions or suspensions.

Polypeptides to be used according to this invention may be synthesized using standard techniques such as those described in Bodansky, M. Principles of Peptide Synthesis (1993) Springer Verlag, Berlin. Automated peptide synthesizers are commercially available (e.g.

Advanced ChemTech Model 396; Milligen/Biosearch 9600). Peptides may be purified by high pressure liquid chromatography and analyzed by mass spectrometry. One or more modifying groups may be attached to such a peptide by standard methods, for example by modification of amino, carboxyl, hydroxyl or other suitable reactive groups on an amino acid side chain or at either terminus of a peptide (e.g. Greene, T.W. and Wuts, P.G.M. Protective Groups in Organic Synthesis (1991) John Wyley & Sons Inc., New York). Polypeptides may also be prepared according to standard recombinant DNA techniques using a nucleic acid molecule encoding the peptide. A nucleotide sequence encoding a desired peptide may be determined pursuant to the genetic code and an oligonucleotide having this sequence may be synthesized by standard DNA synthesis methods (e.g. using automated DNA synthesizer) or by deriving such DNA from a natural gene or cDNA using standard molecular biology techniques such as site-directed mutagenesis, polymerase chain reaction, and/or restriction enzyme digestion. Production of recombinant adenovirus proteins is known in the art, including from literature described herein.

This invention includes the use of nucleic acids encoding proteins and polypeptides to be used in this invention. To facilitate expression of a peptide in a host cell by recombinant DNA techniques, nucleic acids according to this invention may be incorporated into a recombinant vector. Accordingly, this invention also provides such vectors comprising the nucleic acid molecules of this invention. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Vectors may include circular double stranded DNA plasmids and viral vectors. Certain vectors are capable of autonomous replication in a host cell such as vectors of bacterial origin and episomal mammalian vectors. Other vectors such as non-episomal mammalian vectors may be integrated into the genome of a host cell upon introduction into the host cell and thereby may be replicated along with the host cell genome. Certain vectors may be capable of directing the expression of genes to which they have been operatively linked and are referred to as expression vectors.

A nucleotide sequence encoding a polypeptide to be used in this invention may be operatively linked to one or more regulatory sequences selected on the basis of the host cells to be used for expression. This means that the sequences encoding the peptide are linked to a regulatory sequence in a manner that allows for expression of the peptide. Such regulatory sequences may include promoters, enhancers, polyadenylation signals and other expression

control elements such as are described in Goddel; Gene Expression Technology: Methods in Enzymology 185 (1990) Academic Press, San Diego, California. Regulatory sequences may direct constituative expression in many types of host cells or may direct expression only in certain tissues or cells. Regulatory elements may direct expression in a regulatable manner such as only in the presence of an inducing agent. Suitable expression vectors for Adenovirus polypeptides are known in the art, including references referred to herein.

Proteins and polypeptides to be used according to this invention may comprise sequences of amino acids not derived from Adenovirus, including fusion proteins. Fusion proteins may comprise a polypeptide of this invention fused to a peptide sequence that facilitates polypeptide transfer across a cell membrane. Also included in this invention are derivatives of proteins and polypeptides of this invention, including derivatives intended to enhance the immunogenicity, biological activity, or pharmacokinetic properties of the polypeptide or protein. Further, polypeptides of this invention may be modified by labelling or by coupling to another agent intended to facilitate detection or recovery of the polypeptide of this invention. Examples of such labelling include coupling to an enzyme or a detectable label such as a radioactive element. Examples of modification to affect pharmacokinetic properties include modification of N or C termini (e.g. to include an amide group or a D-amino acid) to reduce the ability of a polypeptide of this invention to act as a substrate for a carboxypeptidase or a aminopeptidase, or myristoylation to improve accessibility to a cell interior.

Examples of suitable parenteral administration include intravenous, subcutaneous and intramuscular routes. Intravenous administration can be used to obtain acute regulation of peak plasma concentrations of the drug as might be needed for example to treat acute episodes of airway hyperresponsiveness. Improved half-life and targeting of the drug to the airway epithelia may be aided by entrapment of the drug in liposomes. It may be possible to improve the selectivity of liposomal targeting to the airways by incorporation of ligands into the outside of the liposomes that bind to airway-specific macromolecules. Alternatively intramuscular or subcutaneous depot injection with or without encapsulation of the drug into degradable microspheres eg. comprising poly (DL-lactide-co-glycolide) may be used to obtain prolonged sustained drug release as may be necessary to suppress the development of airway hyperresponsiveness. For improved convenience of the dosage form it may be possible to use an i.p. implanted reservoir and septum such as the Percuseal system available from

Pharmacia. Improved convenience and patient compliance may also be achieved by the use of either injector pens (eg. the Novo Pin or Q-pen) or needle-free jet injectors (eg. from Bioject, Mediject or Becton Dickinson). Prolonged zero-order or other precisely controlled release such as pulsatile release can also be achieved as needed using implantable pumps. Examples include the subcutaneously implanted osmotic pumps available from ALZA, such as the ALZET osmotic pump.

Nasal delivery may be achieved by incorporation of the protein drug into bioadhesive particulate carriers (<200 μm) such as those comprising cellulose, polyacrylate or polycarbophil, in conjunction with suitable absorption enhancers such as phospholipids or acylcarnitines. Available systems include those developed by DanBiosys and Scios Nova.

Oral delivery may be achieved by incorporation of a drug into enteric coated capsules designed to release the drug into the colon where digestive protease activity is low. Examples include the OROS-CT/Osmet.TM. and PULSINCAP.TM. systems from ALZA and Scherer Drug Delivery Systems respectively. Other systems use azo-crosslinked polymers that are degraded by colon specific bacterial azoreductases, or pH sensitive polyacrylate polymers that are activated by the rise in pH at the colon. The above systems may be used in conjunction with a wide range of available absorption enhancers.

Targeted delivery of high doses of a drug to the site of airway hyperresponsiveness can be directly achieved by pulmonary delivery. The lower airway epithelia are highly permeable to wide range of proteins of molecular sizes up to 20 kDa (eg. granulocyte colony stimulating factor). It is possible to spray dry proteins in suitable carriers such as mannitol, sucrose or lactose. Micron-sized particles may be delivered to the distal alveolar surface using dry powder inhalers similar in principle to those designed by Inhale, Dura, Fisons (Spinhaler), Glaxo (Rotahaler) or Astra (Turbohaler) propellant-based metered dose inhalers. Solution formulations with or without liposomes may be delivered using ultrasonic nebulizers. See the following references for further discussion of this topic: McElvaney, *et al.*, J. Clin. Invest., 90, 1296-1301 (1992); and Vogelmeier *et al.*, J. Appl. Physiol., 69, 1843-1848 (1990).

The amount of the pharmaceutical composition to be employed will depend on the recipient and the condition being treated. The requisite amount may be determined without undue experimentation by protocols known to those skilled in the art. Alternatively, the requisite amount may be calculated, based on a determination of the amount of tryptase

which, must be inhibited in order to treat the condition. As the active materials contemplated in this invention are deemed to be nontoxic, treatment preferably involves administration of an excess of the optimally required amount of active agent.

Virus strains and tissue culture. Wild Type Ad5 (Ad5wt) was obtained from the American Type Culture Collection (Rockville, Maryland, USA) and dl739, E3/6.7K-deleted viral mutant (dl739) (Brady *et al.*, 1992) was obtained as a gift from W.S.M Wold. These two Adenovirus group C viruses share a great degree of similarity, but differ in the expression of E3/6.7K protein, which is deleted in dl739 as described previously(Brady *et al.*, 1992). Both viral serotypes were propagated in monolayer culture of A549 cells grown in Minimal Essential Media (Gibco BRL Life Technologies Inc., Gaithersburg, Maryland, USA) supplemented with 10% Fetal Calf Serum (FCS). Two to five days after inoculation with Ad5, cells were freeze/ thawed twice, sonicated for 30s three times and centrifuged at 500xg for 5 min. The supernatant was collected and its viral titer determined by plaque assays on A549 monolayers grown on six well plates. Titers ranged from 10^8 to 10^9 plaque-forming units (pfu)/ml. Control inoculum was prepared from uninfected A549 cells treated in an identical manner to the infected cells.

Inoculation of airway ducts and viral plaque assays. Two groups of 24 mice were anaesthetized with Halothane. One group of mice were infected intranasally with 10^7pfu of Ad5wt in 60 µl of culture media while the other group of mice was infected intranasally with 10^7pfu of dl739 in 60 µl of culture media. In addition, six animals were infected with sterile culture media alone. Six animals from each of the two groups were sacrificed with an overdose of Halothane 2 hours, 1, 3 and 7 days post infection (p.i.). Two sham infected animals were sacrificed on days 1, 3 and 7 days p.i.. The left lung was removed and frozen in liquid nitrogen for use in viral plaque assays. The right lung was inflated with 4% paraformaldehyde in PBS pH7.4 (0.149 M NaCl, 0.012 M Na2HPO4, 0.004M KH2PO4) and embedded in paraffin.

Viral titer. Viral plaque assays were used to quantitate the amount of replicating virus in mouse lungs. Approximately 200mg of lung was homogenized on ice in 1ml sterile MEM with a polytron. The homogenate was spun for 2 min at 10,000g while the supernatant was removed and stored at -70°C. The lung homogenate supernatant titer was determined by plaque assay on A549 cell monolayer cultures grown in MEM/10%FBS on six well plates using decimal dilutions from 10-1 to 10-6 in MEM from the supernatant of animal lung

homogenate from all time groups. Each well was inoculated with 500 µl of diluted supernatant and virus was allowed to adsorb onto the monolayer of A549 cells for 1h. at 37°C An agarose overlay (0.9% agarose, MEM, 2% FCS, and 0.001 neutral red at 37°C) was applied after adsorption. Plaques were counted after 10-14 days and normalized to lung mass and expressed as (log pfu/g lung tissue).

Histologic scoring. Four µm sections of paraffin embedded lung tissue were mounted on glass slides and stained with hematoxylin and eosin. An independent observer, unaware of the experimental treatment of the tissue sections, scored the airway mucosal, airway adventitia and the vascular adventitia for inflammation. The histopathologic grades were 0 - no inflammation, 1 – mild inflammation. 2 – moderate inflammation, 3 – severe inflammation for each feature. The scores for each feature were summed to give a total inflammatory score with maximum being 9 for each animal. A mean inflammatory score was calculated for each animal by dividing the total score by 3. The mean and standard deviation was calculated for each experimental group.

Statistical analysis. Comparisons between the two virus were made for viral titer, inflammatory score and time using a 2-way ANOVA. The level of significance was $p<0.05$.

Plasmid constructs. cDNA for E3/6.7K was obtained by amplifying by PCR the region coding for the E3/6.7K ORF from a vector carrying the Ad2 E3 region (obtained as a gift from W.S.M. Wold). The PCR product was cloned in the Xho1 site of the BPV based cDNA expression vector pBCMGSneo (Karasuyama and Melchers, 1988) and sequenced to ensure accuracy

5'-***ACCACC***ATGAGCAATTCAAGTAACTC (forward primer; Fig. 1)

5'-CC***TTA***TCTTGGATGTTGCCCCCAG (reverse primer; Fig. 1)

To isolate the cDNA for E3/6.7K, the above primers and template DNA purified from HEK-293 cells infected with Ad2 and Ad5, 24hr. post infection were used. The reaction cocktail contained template DNA, 0.5µM of each primer forward and reverse, 250µM of each nucleotide, 5U of Pfu polymerase (Canadian Life Technologies, 2270 Industrial St., Burlington , Ontario) in 1X Pfu Buffer. The reaction conditions are: melting of double stranded DNA at 95°C for 30sec., followed by annealing at 57°C for 30 sec., followed by a 30 sec. ramp to 72 and continued elongation for an additional 30sec.. In most cases 30 cycles of the above PCR reaction produce sufficient DNA for most applications. The newly generated cDNA for E3/6.7K contained modifications (highlighted in bold in Figure 1 and the

sequence of the primers depicted above) which are not found in naturally occurring E3 nucleic acid. Both modifications enhance translation initiation at the start site of E3/6.7K and provide for increased production of the protein in a transformed cell. The naturally occurring form of E3/6.7K is very inefficient. The forward primer provides the start site of E3/6.7K with an optimal upstream Kozak consensus sequence. The reverse primer was modified to replace the naturally occurring TGA-Stop codon with an Ochre-Stop codon (TAA). The latter modification eliminates the start site of E3/19K, which overlaps with the sequence of E3/6.7K in the naturally occurring E3 nucleic acid and results in poor translation of E3/6.7K from the natural sequence.

Generation of stable U937 cell lines expressing E3/6.7K. U937 human histiocytic lymphoma cells (Sundstrom and Nilsson, 1976) obtained from ATCC (CRL 1593) were maintained in RPMI 1640, 10% FCS, 2mM L-glutamine, 10mM HEPES, 100U/ml penicillin and 100µg/ml streptomycin in an atmosphere of 5% CO2 and 100% humidity. Cells were transfected with the appropriate construct by using the DMRIE-C cationic lipid reagent available from Life Technologies using the manufacturer's protocol. Transfected cells were maintained in medium containing geneticin G-418 sulphate at a final concentration of 800µg/ml. Media and supplements were purchased from Life Technologies. Subclones of the transfected cell lines were generated by serial dilution and examined for expression of E3/6.7K by Northern Blotting. The expression of E3/6.7K mRNA was very similar in all the clones examined. All the G-418 resistant cells that survived the selection procedure were pooled and used for the *in vitro* assays, in order to avoid clonal variations known to arise in U937 cells.

Labelling, immunoprecipitation and Western Blotting of proteins from transfected cells. U937 cells transfected with vector or with vector carrying E3/6.7K were grown in suspension until they were growing exponentially. 10^8 cells were harvested, washed and intracellular pools of cysteine and methionine were depleted by incubation in prewarmed methionine/cysteine-free essential media without FCS for one hour at 37°C at a concentration of $5x10^6$ cells/ml. A total of $2x10^7$ cells were labelled for one hour in prewarmed methionine/cysteine-free media containing 0.5mCi/ml [35S]-Cysteine and 0.2 mCi/ml (Amersham) [35S]-Methionine (Amersham) at a concentration of $5x10^6$ cells /ml. Cells were washed and then lysed on ice in freshly made lysis buffer containing 1% TritonX-100, 1% BSA (bovine serum albumin), 1mM iodoacetamide, 1mM PMSF, 2.5TIU/ml aprotinin,

0.01M Tris pH8.0, 0.14M NaCl. Samples were counted by TCA precipitation and approx. 10^7cpm of each sample was precleared O/N using protein A-Sepharose CL-4B, the supernatant was immunoprecipitated using a polyclonal rabbit antiserum raised against the C-terminal portion of E3/6.7K and protein A-Sepharose. The pellet was denatured in SDS/sample buffer and loaded on a Tricine-SDS PAGE gel, 16.5%T, 3%C separating gel with a 10%T, 3%C spacer gel (Schagger and von Jagow, 1987). Alternatively, cell lysate equivalent to 105 cells was denatured in SDS-PAGE loading buffer and loaded on 10% glycine SDS-PAGE gel system, separated and blotted onto a Immobilon-P PVDF membrane (Millipore) and probed with cPLA2 rabbit polyclonal antiserum (Cayman Chemical). The signal was detected via horse radish peroxidase-conjugated, goat antirabbit antiserum and by chemiluminescence using the ECL kit (Biorad).

Arachidonic acid release assays. Cells were grown at low density in 10% Hyclone FCS, RPMI 1640, 2mM L-glutamine, 10mM HEPES for several days then harvested and washed twice in PBS, 1% BSA. Approximately $5x10^6$ cells ($5x10^5$ cells/ml) were labelled for 20hrs in same media as above supplemented with 0.4 µCi/ml [3H] arachidonic acid [5,6,8,9,11,12,14,15-3H(N)] (0.1mCi/ml stock; New England Nuclear). Cells were washed twice in RPMI 1640, 0.2%BSA and incubated for one hour in the wash media in order to minimize the spontaneous release of [3H] arachidonic acid. The number of cells was normalized in all cell lines and 400µl of cell suspension was aliquoted in each well of a 24 well plate containing 100µl of treatment media ($2x10^5$ cells/well corresponding to $1.4x10^3$ counts/well). The assay was set up in triplicate and the cells were stimulated either with media alone or with 20ng/ml human rTNF-α (2000U/ml) (Boehringer, Mannheim), or with 10µg/ml cycloheximide or with a combination of 20ng/ml TNF-α and 10µg/ml cycloheximide. After 20 hours of treatment the cells were centrifuged and 100µl of supernatant out of 500µl total was mixed with 3ml scintillation fluid and counted. For each cell line three samples were lysed in lysis buffer and the lysate was used to determine the total counts of incorporated [3H] Arachidonic Acid. The counts per minute of released [3H] arachidonic acid were expressed as a percentage of the average of total incorporated [3H] arachidonic acid.

Annexin V-FACS apoptosis assay. Annexin V-FITC (PharMingen) was used to determine the binding of Annexin V to externalized phosphatidyl serine. The protocol followed was based on the manufacturers Annexin V-FITC staining protocol. Cells were

grown at low density in 10% Hyclone FCS, RPMI 1640, 2mM L-glutamine, 10mM HEPES for several days then $5x10^6$ cells were harvested and washed twice in PBS. Cells resuspended in above media were treated for 7 hours with media alone or with 100ng/ml (10,000 U/ml) human rTNF-α or with 200 μg/ml cycloheximide or with a combination of 100 ng/ml TNF-α and 200 μg/ml cycloheximide. The cells were resuspended at $1x10^6$ cells/ml in 1xBinding Buffer (10mMHepes/NaOH, pH7.4, 140mM NaCl, 2.5mM CaCl2). $1x10^5$ cells (100μl of above suspension) were combined with 5μl of Annexin V-FITC. One sample of cells was not stained and used to set up the baseline fluorescence. The cells were examined with a fluorescence-activated cell sorter (FACS) on a Beckton Dickson FACS Analyzer.

Production of Ad vectors for gene therapy. The backbone for gene therapy is based on the SV5 backbone previously described (Chen 1997 PNAS). This backbone has been successfully used to transduce *in vivo* the dystrophin gene. The backbone lacks the E1 and E2 region. Without these two regions the SV5 Ad vector is replication defective and therefore safer to use as well as it elicits a reduced inflammatory response. The cDNA encoding E3/6.7K under the control of the actin promoter and the CMV enhancer was added to SV5 and used to rescue a new vector called SV5-6.7, which will incorporate E3/6.7K as an immunomodulatory protein.

Creation of Producer Cells resistant to apoptosis. The creation of hybridoma, Chinese hamster ovary (CHO) or insect cells that are resistant to apoptosis will follow the same procedure as the transfection of U937 cells outlined with the following exception. At the end of the selection in G-418 the cells are sorted or clonally expanded in order to screen for the expression of the protein of interest.

It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described aspects of the present invention, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

EXAMPLES

E3/6.7K Results in more Persistent Viral Titers and a reduction of the inflammatory response. The presence of E3/6.7K results in more persistent viral titers

during the course of infection by comparing mice infected with a E3/6.7K deletion virus (dl739)(Brady *et al.*, 1992) with mice infected with the wild type virus (Ad5wt). The titers of dl739 (E3/6.7K deleted) were significantly higher than Ad5 wild type (Ad5wt) one day after inoculation ($p<0.001$). Over time, the titers of dl739 decreased as the virus was cleared ($p<0.001$). In contrast Ad5wt titers did not change significantly over the 7 day experimental period. The rapid reduction of dl739 over the seven day period is attributed to a strong host response due to the increased inflammation in the absence of E3/6.7K. Inflammation of the perivascular region of the blood vessels and the adventitia of the airways was greater in animals infected with dl739 than in animals infected with Ad5wt over the seven days experimental period ($p=0.025$). There was also a significant increase in inflammation from day three to day seven for both types of viruses ($p=0.029$).

TNF-α Mediated Arachidonic Acid Release Is Reduced in the Presence of E3/6.7K. E3/6.7K can affect the cellular response to inflammatory cytokines. A U937 cell line was transfected with the cDNA for E3/6.7K and expression of E3/6.7K was confirmed using immunoprecipitation with a polyclonal rabbit antiserum raised against an E3/6.7K C-terminal derived peptide and SDS-PAGE electrophoresis. The U937 cells transfected with E3/6.7K cDNA (U937-E3/6.7K) decreased [3H] arachidonic acid release by 50% when compared with U937 cells transfected with vector alone (U937neor) when stimulated with TNF-α. When the stimulus was increased by the addition of TNF-α and cycloheximide (CHX), a protein synthesis inhibitor synergistic with TNF-α, U937-E3/6.7K were still able to reduce the release of [3H] arachidonic acid by 60% when compared to U937neor. The presence of E3/6.7K reduces the levels of inducible release of [3H] arachidonic acid during TNF-α stimulation.

Apoptosis Induced by TNF-α is Reduced in the Presence of E3/6.7K. TNF-α induced apoptosis was assayed by measuring by measuring the externalization of phosphatidyl serine using FITC labelled Annexin V (Martin *et al.*, 1995). Cells expressing E3/6.7K show a 55% reduction in percentage of apoptotic cells compared with U937neor following stimulation with TNF-α. The U937-E3/6.7K cells show a 65% reduction in apoptosis compared to U937neor following an augmented stimulation with a combination of TNF-α and CHX. The presence of E3/6.7K decreased the apoptotic response in U937 cells upon stimulation with TNF-α or a combination of TNF-α and CHX.

In the Presence of E3/6.7K, cPLA2 Is Cleaved to a 78kDa Form Following TNF-α Induction. The expression of cPLA2 in U937 cells following induction with TNF-α was assayed. The cPLA2 antiserum recognized two forms of the enzyme: one larger form of approximately 110kDa; and a second form of 78kda. There was a noticeable difference between U937neor cells and U937-E3/6.7K with regards to the ratio of the 110kDa versus the 78kDa forms of cPLA2. While TNF-α does not seem to alter this ratio in U937neor (cells where the predominant form migrates as a 110kDa protein) in U937-E3/6.7 K cells following induction with TNF-α the most predominant form of cPLA2 is 78kDa. The antisera was raised against a peptide corresponding to residues 443-462 of the cPLA2 sequence, therefore the only fragment detected by immunoblotting following cleavage is the 78kDa fragment corresponding to the 1-522 amino acid sequence of cPLA2 as isolated from U937 cells (Sharp *et al.*, 1991).

Although various aspects of the present invention have been described in detail, it will be apparent that changes and modification of those aspects described herein will fall within the scope of the appended claims. All publications and patent documents referred to herein are incorporated by reference.

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